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| <b>(54) Title:</b> DOPAMINERGIC NEUROTROPHIC FACTOR FOR TREATMENT OF PARKINSON'S DISEASE<br><br><b>(57) Abstract</b><br><br>Dopaminergic Neurotrophic Factor (DNTF), derived from cells of the peripheral nervous system, is administered to pa-<br>tients suffering from Parkinson's Disease in an amount effective to facilitate survival of substantia nigra dopamine nerve cells.   |           |   |

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DOPAMINERGIC NEUROTROPHIC FACTOR FOR TREATMENT  
OF PARKINSON'S DISEASE

BACKGROUND OF THE INVENTION

The present invention relates to a composition of matter, derived from cells of the peripheral nervous system, comprising dopaminergic neurotrophic factor, to a pharmaceutical preparation containing the  
5 dopaminergic neurotrophic factor, and to its use in the treatment of Parkinson's disease.

Parkinson's disease is a neurodegenerative disorder of the basal ganglia affecting specific  
10 populations of neurons in the central nervous system. Symptoms of Parkinson's disease include tremor at rest, muscular rigidity, akinesia and bradykinesia.

The primary neuropathology associated with this disorder is the progressive and persistent loss of  
15 dopaminergic neurons originating in the substantia nigra and projecting into the striatum. This, in turn, leads to a substantial decrease in the enzymes responsible for the synthesis of the neurotransmitter, dopamine. The subsequent decrease in dopamine  
20 synthesis correlates with the onset and severity of the above-noted symptoms.

Evidence indicating that the loss of dopaminergic neurons is causally connected with the symptoms associated with Parkinson's disease was found in 1983.  
25 Specifically, certain drug abusers who injected a toxin, known as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), as a heroin substitute developed signs of parkinsonism soon after injection. It was subsequently determined that MPTP is converted to a  
30 form (MPP+) that accumulates in substantia nigra dopamine neurons where it acts as a toxin destroying these neurons. The resultant loss of dopaminergic neurons was found to mimic the neuropathology observed in Parkinson's disease.

Studies have shown that Parkinson's disease, as well as other neurodegenerative disorders such as Alzheimer's disease and amyotrophic lateral sclerosis (ALS), may occur due to the loss or decreased availability of a neurotrophic substance specific for a particular population of neurons affected in each disorder. As used herein, "neurotrophic factor" refers to a substance or combination of substances whose primary function is to increase and/or maintain the survival of a neuronal population, but may also affect the outgrowth of neuron processes (neurite-promoting factors), and the metabolic activity of a neuron. The specific neurotrophic factor is synthesized, stored, and/or released from the target area of the degenerating neurons, bound and internalized by specific receptors, and transported in a retrograde fashion to the neuron body where it exerts its trophic effects well into adulthood. It may be the loss of such specific neurotrophic factors which is responsible for age-related declines in cell survival and/or function. While the cellular source remains unclear, there is evidence to suggest that neurons and glia are both capable of secreting neurotrophic factors.

Several putative neurotrophic factors effecting specific neuronal populations in the central nervous system have been reported. For example, it is postulated that Alzheimer's disease is the result of the loss or decreased availability of nerve growth factor (NGF), a polypeptide of approximately 13,000 dalton molecular weight in the monomer form. NGF is known to increase the survival, function and regeneration of cholinergic neurons in the basal forebrain. This population of cholinergic neurons has been shown to shrink and/or die in patients having Alzheimer's disease, and may be the primary neuronal

defect responsible for the profound cognitive deficits associated with Alzheimer's disease. Recent studies have demonstrated that NGF is synthesized and released from the target areas of these cholinergic neurons, the hippocampal formation and neocortex. Thoenen, H. et al., Rev. Physiol. Biochem. Pharmacol. 109:145-178 (1987); and Whittemore, S.R. et al., Brain Res. Rev. 12:439-464 (1987). Insofar as is known, there is no conclusive evidence that a loss of NGF production is the primary cause of degeneration of the basal forebrain cholinergic neurons. However, it has been proposed to treat Alzheimer patients by administering exogenous NGF, in order to increase the survival of degenerating neuronal populations.

At the present time, the therapy of choice for patients having Parkinson's disease is through stimulation of dopamine receptors in the striatum, which is the target area of substantia nigra neurons. This is achieved through "precursor drug therapy", involving the administration of  $\beta$ -(3,4-dihydroxy phenyl)- $\alpha$ -alanine(L-DOPA/LEVODOPA), which passes the blood-brain barrier and is converted to dopamine. While this pharmacological approach is initially effective, L-DOPA treatment often becomes less effective over time and in many cases the patients' symptoms worsen.

Numerous neurotrophic factors, in addition to NGF, which produce biological effects in the central nervous system have been reported, and these will be more specifically discussed hereinbelow. Insofar as is known, however, there is no currently available method for rescuing degenerating dopaminergic neurons in the substantia nigra. In addition, the conditions responsible for the onset of the degeneration of these nerve cells have not been elucidated. Thus, there is

currently no clearly effective cure for Parkinson's disease.

#### SUMMARY OF THE INVENTION

5 In accordance with one aspect of the present invention, there is provided a composition of matter for treatment of Parkinson's disease, which comprises a purified and concentrated form of dopaminergic neurotrophic factor (DNMF) derived from cultured cells of the mammalian peripheral nervous system. DNMF has  
10 a molecular weight of less than 10,000 daltons and exhibits a neurotrophic effect on substantia nigra dopamine nerve cells. Among the notable properties of DNMF is its ability to increase the survival time of fetal non-mitotic dopamine nerve cells in culture and  
15 to increase in vivo expression of tyrosine hydroxylase in substantia nigra dopamine nerve cells exposed to said factor, while having no appreciable effect on neurite outgrowth.

20 In accordance with another aspect of this invention, there is provided a pharmaceutical preparation for the treatment of Parkinson's disease which comprises, as the active agent, the aforesaid DNMF in an amount sufficient to increase the survival and function of dopamine nerve cells located in the  
25 substantia nigra and projecting to the striatum, and, possibly, to cause regeneration of these cells.

In accordance with a further aspect of the present invention, there is provided a method for treating patients having Parkinson's disease, which  
30 comprises administering to such patients the above-described DNMF.

The present invention represents a potentially important alternative to current therapy used for treatment of Parkinson's disease. The precursor drug  
35 therapy (L-DOPA) now in use does not provide a cure for Parkinson's disease, but rather is a method of

treatment that may become ineffective and even detrimental with prolonged use. It is anticipated that treatment of Parkinson's patients with the dopamine neurotrophic factor of the invention will  
5 inhibit or halt the progress of the disease by reducing the degeneration and dysfunction of substantia nigra nerve cells. In addition, dopamine neurotrophic factor treatment may be useful in transplantation strategies where dopamine cells are  
10 transplanted as a means of replacing lost dopamine function. Specifically, dopaminergic neurotrophic factor may be administered in conjunction with central nervous system grafts of dopamine-synthesizing tissue in order to enhance the survival and function of the  
15 grafted tissue.

The chemical nature of the dopaminergic neurotrophic factor of the invention that is responsible for the observed activity against Parkinson's disease has not been clearly defined. It  
20 may be a single chemical substance or a mixture of substances. Accordingly, the singular of the terms "agent", "component", "ingredient", or the like, as used herein in reference to the DNTF, also includes the plural.

25 Although numerous neurotrophic factors having biological effects in the central nervous system have previously been reported, including factors derived from cells of the peripheral nervous system, none of the factors obtained heretofore are believed to  
30 exhibit physical or biological properties identical to the dopaminergic neurotrophic factor of the invention, as will appear hereinbelow. Moreover, attempts previously made to recover a DNTF from the striatum, prompted by evidence supporting its presence there,  
35 has not led to the successful isolation of such factor

Tomoizawa, Y et al., Brain Research, 399:111-124 (1986).

DETAILED DESCRIPTION OF THE INVENTION

As noted above, the primary symptoms of  
5 Parkinson's disease are caused by a defect in a  
specific neurotransmitter system, the nigrostriatal  
dopamine system. Specifically, dopamine neurons in  
the substantia nigra degenerate, resulting in the loss  
of dopamine input to the striatum and the onset of  
10 characteristic movement abnormalities.

One possible explanation for the degeneration of  
the dopamine-containing nerve cells is that a specific  
dopamine neurotrophic factor becomes ineffective,  
unavailable or is no longer synthesized by the target  
15 regions in the striatum. In addition, most  
neurotrophic factors function through specific,  
membrane-bound receptors located on presynaptic  
terminals. Alterations in the function of these  
receptors would tend to render the neurotrophic factor  
20 ineffective. In a normal, healthy individual,  
dopamine neurotrophic factor is released from the  
target region of these dopamine-containing substantia  
nigra nerve cells in the striatum. This factor is  
recognized and bound by specific receptors,  
25 internalized as a complex, and transported in a  
retrograde fashion to the cell body of the nerve cell  
where it functions to maintain dopamine neuron  
survival and normal homeostatic function. In the case  
of Parkinson's disease, by comparison, the striatum  
30 may no longer be providing an adequate supply of the  
dopaminergic neurotrophic factor, resulting in  
dopamine nerve cells that are no longer able to  
function adequately and may eventually die due to the  
loss of the dopaminergic neurotrophic factor.

35 The DNTF used in the practice of this invention  
is a soluble protein of molecular weight less than

10,000 daltons which is extractable from cells of the peripheral system. While the peripheral nerve is not a target of the central nervous system dopamine nerve cells, the cells associated with the peripheral nerve are known to synthesize and secrete a number of different trophic factors, e.g. NGF, especially following denervation.

The extraction of DNTF is performed on peripheral nerve preparations that have been incubated in serum-free culture medium. Protease inhibitors, such as leupeptin, may be included in the serum-free culture medium in order to minimize the degradation of proteins secreted by the peripheral nerve segments. DNTF is recoverable from the culture medium using conventional molecular weight exclusion techniques. Extraction has been satisfactorily performed using centrifugation filters with molecular weight exclusion capabilities. For example, Centricon®-10 and -30 (Amicon) centrifuge filtration tubes may be used to obtain fractions from the peripheral nerve preparation. This procedure allows for the isolation of a first fraction comprising molecules of molecular weight less than 10 kilodaltons (Centricon-10) and a second fraction comprising molecules of molecular weight less than 30 kilodaltons (Centricon-30). Dopamine neurotrophic activity is exhibited by the filtrate obtained from the Centricon-10 tubes, indicating that dopaminergic neurotrophic factor is of a molecular weight lower than 10 kilodaltons. The second fraction (molecular weight range between 10,000-30,000 daltons) includes a molecule or molecules that cause(s) dramatic increases in the outgrowth of neuron processes in dopamine-containing nerve cells in culture.

Additional conventional processing steps may be implemented to further isolate and purify the desired

product. Because the DNTF is a relatively small protein, it may be isolated using reverse phase, high-performance liquid chromatography (RPHPLC). Alternatively, the dopaminergic neurotrophic factor  
5 may be isolated using a variety of other techniques including ion exchange chromatography or absorption chromatography. Large scale isolation may be performed using affinity chromatography, preferably with an appropriate monoclonal antibody having binding  
10 affinity for the DNTF. The desired product is then sterilized, lyophilized and its neurotrophic activity determined using the culture bioassay described below (see Example 2).

Sources of DNTF would include pure cultures  
15 of cells associated with the aforementioned peripheral nerve preparation, such as Schwann cells and/or fibroblasts. These cell populations may be isolated from culture medium to homogeneity and analyzed for DNTF. The presence of DNTF in these culture preparations  
20 would allow for its large scale preparation.

The dopamine neurotrophic activity of the recovered material is readily determined via bioassay. One method of assaying for neurotrophic activity is to determine biological activity in cultures of  
25 dopaminergic nerve cells. The DNTF of the invention has been found to exhibit selective survival and survival-related effects, i.e. production of dopamine-synthesizing enzymes, on dopamine nerve cells using the culture bioassay. Other measures of  
30 dopaminergic neurotrophic activity, besides survival, include cell growth and metabolic functions associated with normal homeostatic function, such as high affinity dopamine uptake. Following incubation with fractions exhibiting dopaminergic neurotrophic  
35 activity, dissociated cell cultures are stained using tyrosine hydroxylase immunocytochemistry. Tyrosine

hydroxylase is an enzyme necessary for the production of dopamine. Thus, by using antibodies to tyrosine hydroxylase, dopamine-containing nerve cells may be identified. Once the dopamine-containing nerve cells are identified, measures of cell size can be performed on culture treated with DNTF, as opposed to control treated cultures.

Changes in the levels of tyrosine hydroxylase messenger RNA can also provide a measure of dopamine neuronal function. Recent advances in molecular biology, such as in situ hybridization, permit quantitative analysis of single genes in single neurons. Such techniques make it possible to study the effects of dopaminergic neurotrophic factor on tyrosine hydroxylase gene expression in vitro and in vivo and are currently being implemented toward that end.

Once dopamine is released from the presynaptic terminal, it is degraded by monoamine oxidase or taken up again into the presynaptic terminal by a high affinity uptake mechanism. Using radioactive ( $^3\text{H}$ ) dopamine, the high-affinity uptake of dopamine can be determined in cultures treated with dopaminergic neurotrophic factor or control solutions. Increases in dopamine uptake can indicate increased dopamine synthesis and release, a measure of metabolic function in such nerve cells.

Experiments have been performed, both in vitro and in vivo which demonstrate the neurotrophic effect of DNTF on substantia nigra dopamine nerve cells and its potential for effectively treating Parkinson's Disease. The nature of these experiments and their results are described hereinbelow.

Several experiments have been performed in vivo using rats that have sustained unilateral damage to the nigro-striatal pathway. This damage results in a

massive loss of dopamine input to the striatum, and a behavioral syndrome consisting of amphetamine-induced rotation towards the side of damage. This rotation effect has become a classic model for screening dopamine-related compounds. Indeed, it has been shown in numerous laboratories that transplantation of fetal dopamine neurons can cause reversal of this behavioral deficit. Facilitation of this behavioral recovery over time can be accomplished using co-grafts of peripheral nerve capable of secreting DNTF and mesencephalic dopamine synthesizing cells. It appears that the peripheral nerve secretions (including DNTF) continue to effect the dopamine cells in the host following transplantation.

Other experiments conducted to date include the transplantation of peripheral nerve segments into aging test animals with the compromised dopamine system, i.e. decreased number of dopamine-containing neurons and decreased dopamine synthesis and content. The peripheral nerve graft greatly increases tyrosine hydroxylase staining in remaining substantia nigra neurons, as well as the number of tyrosine hydroxylase-containing nerve fibers.

These experimental results indicate that DNTF is most likely a soluble factor released in vitro and in vivo by peripheral nerves, which may be transported in a retrograde fashion to the cell bodies of substantia nigra neurons, so as to enhance their survival and function.

As noted above, numerous neurotrophic factors exhibit biological effects in the central nervous system, including factors derived from the peripheral nervous system. Some of the well-characterized factors are listed below in Table 1. DNTF is distinctly different from all of the neurotrophic

factors listed in Table 1, notwithstanding that it shares certain characteristics with some of them.

Table 1. Purified and partially-purified neurotrophic factors, their effects in the central nervous system, and selected physical properties

|  | <u>FACTOR</u>                            | <u>EFFECTS</u>                                     | <u>PROPERTIES</u>    |
|--|--|--|----------------------|
|  | Nerve growth factor (NGF)                | survival of cholinergic neurons, neurite induction | MW 13,000<br>pI 10.0 |
|  | Ciliary neurotrophic factor (CNTF)*      | survival, neurite outgrowth                        | MW 20,400<br>pI 5.0  |
|  | Brain-derived neurotrophic factor (BDNF) | survival, (additive with NGF)                      | MW 12,300<br>pI 10.1 |
|  | Insulin-like growth factor-II (IGF-II)   | survival, neurite outgrowth                        | MW 7,100             |
|  | Basic fibroblast growth factor (bFGF)    | survival, neurite outgrowth                        | MW 16,400<br>pI 9.6  |
|  | Acidic fibroblast growth factor (aFGF)   | neurite outgrowth                                  | MW 15,800<br>pI 5.0  |
|  | Striatal-derived neuronotrophic factor   | survival of dopamine cells, neurite outgrowth      | MW 14,000            |

|                  |                           |          |
|------------------|---------------------------|----------|
| Striatal extract | survival of dopamine      | MW 1500- |
| factors          | cells, neurite outgrowth, | 2200     |
|                  | dopamine uptake           |          |

5 The asterisk indicates a factor derived from cells of the peripheral nervous system.

One of the characteristics of a true neurotrophic factor is the ability to increase the survival of central nervous system (CNS) neurons. Based on this  
10 criterion, aFGF, listed in Table 1, is not a true neurotrophic factor, but rather may be regarded as a "neurite-promoting" factor. Similarly, numerous other factors, including, for example, fibronectin, collagen and laminin, are able to promote neurite outgrowth,  
15 without appreciably influencing the survival of the neuronal population.

Among the neurotrophic factors, listed in Table 1, CNTF is synthesized and released by denervated peripheral nerves and influences the survival and  
20 outgrowth of numerous neuronal populations including ciliary neurons, sympathetic neurons, dorsal root ganglia and some centrally-derived neurons. DNTF does not share such neurite outgrowth properties and is lower than CNTF in molecular weight (less than 10,000  
25 for DNTF as compared to 20,400 for CNTF).

Another factor that may be secreted by peripheral nerves is bFGF. Although bFGF may be considered a true neurotrophic factor, at least three characteristics serve to distinguish bFGF from DNTF.  
30 First, the molecular weight of bFGF is greater than 10,000 daltons, while the molecular weight of DNTF is less than 10,000 daltons. Second, bFGF promotes survival and neurite outgrowth in cultured neurons, while DNTF appears not to affect neurite outgrowth.  
35 Third, bFGF contains a heparin sulfate binding domain. Fractions of peripheral nerve conditioned medium that

have been run over a heparin sulfate column (removing bFGF from the fraction) continued to exhibit neuron survival and neurite outgrowth in cultured dopamine neurons. These data indicate that DNTF is not related to bFGF.

Gangliosides, a family of glycosphingolipids that are present in nerve tissues, may also be secreted by peripheral nerves. While there is no evidence to indicate that gangliosides function as a survival or neurotrophic factor, it appears that the presence of gangliosides may potentiate neurotrophic activity. For example, gangliosides have been shown to potentiate the effects of NGF on cultured basal forebrain cholinergic neurons. In addition, ganglioside treatment has been shown to enhance the regeneration (but not survival) of substantia nigra dopamine neurons following damage. Thus, the effects of gangliosides are not as specific as DNTF, and require the presence of other appropriate trophic influences to be effective.

A recent report describes a peripheral nerve-derived soluble factor(s) that increases the survival and neurite outgrowth of sensory neurons in culture. Windebank, A.J. et al., Brain Research, 385:197-200 (1986). While this report intimates that the factor described therein is novel, the molecular weight and biological properties given are similar to CNTF, listed in Table 1 above.

Considering the factors listed in Table 1 recovered from the striatum, DNTF differs from striatal-derived neurotrophic factor in that DNTF does not appreciably influence neurite outgrowth and its molecular weight is less than 14,000. Moreover, it has been suggested that striatal-derived neurotrophic factor may not be unique, but in fact exhibits properties not unlike those of BDNF and bFGF. Dal.

Toso, R. et al., J. Neurosci., 8:733-745 (1988).

There are other factors found in the striatum that fall within the molecular weight range of 1,500-2,200 daltons. These factors, however, are also found in high concentrations in non-dopaminergic brain regions, such as the hippocampus, amygdala and cerebral cortex, and also influence the high affinity uptake of gamma-amino-n-butyric acid (GABA). These data indicate that striatal extract factors may not necessarily be specific to dopamine neurons.

Moreover, the observation that factors derived from striatal extracts affect neurite number and length is indicative that DNTF and the striatal extract factors are not the same. The use of striatal factors as a diagnostic and therapeutic tool in the treatment of Parkinson's Disease is the subject of a separate patent application. See U.S. Patent application Serial No. 444, 293, filed November 24, 1982, and related applications.

Unlike DNTF, NGF does not exhibit a neurotrophic effect on substantia nigra dopamine nerve cells and promotes neurite induction. The molecular weight of NGF is also higher than that of DNTF. DNTF is similarly distinguishable from BDNF on the basis of their relative molecular weights.

In view of the molecular weight of DNTF, as well as the specific effect on neuron survival, but not on neurite outgrowth, all of the known neurotrophic factors listed in Table 1 above are plainly distinguishable from DNTF, with the sole exception of IGF-II, which has a molecular weight of 7,100. IGF-II is produced in the central nervous system almost exclusively in the astroglia. The role of IGF-II in the peripheral nervous system appears to be related to synapse formation and denervated-induced fiber growth during development and regeneration. Specifically,

IGF-II levels are highest in the target region (muscle fiber) during pre-and early post natal development. Transection of the sciatic nerve also results in increased IGF-II levels in mature denervated muscle fibers. IGF-II has been shown to increase the survival of NGF-sensory and sympathetic neurons in culture. However, direct evidence for IGF-II as a survival factor in the central nervous system is lacking.

Another insulin-related growth factor, IGF-I also is present in the central nervous system and is synthesized in neuronal and non-neuronal cells. IGF-I, which has a molecular weight of about 7,600 daltons, has been shown to undergo retrograde transport in the rat sciatic nerve and may play a role in peripheral nerve regeneration. In addition, IGF-I can act as a survival factor for cortical neurons in transferrin-supplemented medium. At the present time, no effects of IGF-I on survival or neurite outgrowth of cultured dopamine neurons has been reported. Moreover, a recent study has shown that binding sites for IGF-I in the central nervous system are associated with cholinergic, and not dopaminergic brain regions. Araujo, D.M. et al., Brain Res., 484:130-138 (1989). Therefore, given the published results of this study and our own test results, it appears that DNTF is not related to the insulin family of growth factors.

In sum, while there are numerous neurotrophic factors that have biological activities in the central nervous system, as set forth in Table 1 above, the apparent differences in properties between such factors and DNTF provides compelling evidence of the uniqueness of DNTF.

DNTF may be conveniently formulated for administration with a biologically acceptable medium, such as water, buffered saline, polyol (for example,

glycerol, propylene glycol, liquid polyethylene glycol and the like) or suitable mixtures thereof. The optimum concentration of the active ingredient(s) in the chosen medium can be determined empirically, according to procedures well known to medicinal chemists. As used herein, "biologically acceptable medium" includes any and all solvents, dispersion media, and the like which may be appropriate for the desired route of administration of the pharmaceutical preparation. The use of such media for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient of DNTF, its use in the pharmaceutical preparation of the invention is contemplated.

It is especially advantageous to formulate the pharmaceutical preparation in dosage unit form for ease of administration and uniformity of dosage. "Dosage unit form" as used herein, refers to a physically discrete unit of the pharmaceutical preparation appropriate for the patient undergoing treatment. Each dosage should contain the quantity of active ingredient calculated to produce the desired therapeutic effect in association with the selected pharmaceutical carrier. The appropriate dosage unit to be administered for facilitating survival of substantia nigra dopamine nerve cells may be routinely determined by those skilled in the art. It is expected that the standard dosage unit will contain less than a milligram of the active ingredient.

The pharmaceutical preparation is preferably administered parenterally, e.g. by introduction into the central nervous system of the patient. Such administration may be accomplished by intracerebroventricular infusion. Patients may also be treated with DNTF by transplanting into the

striatum cells of the peripheral nervous system capable of releasing DNTF. Such cells may be cotransplanted with dopamine-synthesizing cells of the central nervous system, such as mesencephalic dopamine synthesizing cells. The treatments just described may also be administered in conjunction with one another. For example, dopamine synthesizing cells of the central nervous system may be transplanted into the striatum of a patient who is simultaneously being administered the pharmaceutical preparation of the invention. Non-parenteral routes may also be useful in administering DNTF, including oral, intranasal, rectal as well as ophthalmic administration.

The pharmaceutical preparation of the invention may be administered at appropriate intervals, until the symptoms of the disease are no longer evident, after which the dosage may be reduced to a maintenance level. The appropriate interval of administration in a particular case would normally depend on the condition of the patient. As used herein, the term "patient" includes both humans and animals.

The following examples are provided to describe the invention in further detail. These examples are intended to illustrate and not to limit the invention.

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Example 1 - Isolation and Purification of DNTF

Rat sciatic nerves (approximately 2.5-3.0 cm in length) are placed in 1.0 ml. of sterile, serum-free culture medium containing protease inhibitors and incubated at 37°C in a humid environment containing 95% air and 5% CO<sub>2</sub>. After three days of incubation, the conditioned medium was removed, frozen at -20°C and the culture medium was replaced with new medium. The removal and replacement process was repeated for two more cycles, so that 3.0 ml of conditioned medium was obtained. The conditioned medium was then placed

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into Centricon®-10 and -30 centrifuge tubes and centrifuged for 1 1/2 hours at 4°C. A total of 3 fractions were isolated using this technique. A first fraction contained compounds of molecular weight of 10,000 daltons or less and included the DNTF. A second fraction contained compounds of molecular weight of 30,000 daltons or less, which included substances that dramatically increased the outgrowth of neurite processes, i.e. neurite number, length and branching, in dopamine-containing neurons and culture. A third fraction comprised compounds of molecular weight in excess of 30,000 daltons which included substances which exhibited a neurotoxin-like effect on dopamine-containing neurons. Because DNTF is found in the fraction recovered in the Centricon-10 centrifuge tube, isolation of DNTF can be initiated using this procedure. The DNTF-containing fraction was sterilized by passage through a 0.2 um filter, lyophilized and subjected to neurotrophic activity determination by culture bioassay.

#### Example 2 - Tissue Cultures

Dissociation cultures of dopamine nerve cells were obtained using standard protocols. Specifically, 0.2-0.4 mm pieces of rat ventral mesencephalon (which included A8-A10 dopamine nerve cells) were dissected from embryonic day 13-16 rats. At this stage of development, the dopamine nerve cells were post mitotic, but did not yet innervate the striatum. Dissociated cell cultures were prepared by triturating the tissue in the presence of DNase (1 mg/ml) and trypsin (0.25 mg/ml). Cells were washed in Opti-MEM medium (Gibco) supplemented with 10% fetal bovine serum (FBS) and then plated in 16 mm diameter plastic wells at a density of 500,000 cells per well containing 1.0 ml Opti-MEM and 10% FBS. Cells were

allowed to equilibrate in this solution for 72 hours, at which time the cells were switched to 1.0 ml serum-free Opti-MEM containing a 100 ul solution of DNTF solution. These cells were maintained at 37°C in 95% air-5% CO<sub>2</sub> for a period of 7 days, with the DNTF-containing culture medium being replaced every other day. Thus, the cells received three treatments of DNTF-containing culture medium.

10           Example 3 - DNTF Activity In Vitro

Cultures of mesencephalic nerve cells were stained for tyrosine hydroxylase to identify dopaminergic nerve cells. Cultures treated for 10-14 days with the first fraction obtained in Example 1 exhibited a 1.8-8.0 fold increase in dopamine cell number compared to control treated cultures. Cultures treated with the second fraction obtained in Example 1 also exhibited an increase in dopamine cell survival, but not to the same extent as that observed for the first fraction (1.5 for the second fraction, as compared with 1.8-8.0 for the first fraction). This is probably due to the dilution of the DNTF in the higher molecular weight fraction. However, extensive neurite outgrowth was observed in cultures that were treated with the second fraction from Example 1, as compared with all other treatments. While this neurite-promoting factor has not been conclusively identified, it exhibits properties similar to CNTF (see Table 1 above), which is found in relatively high concentrations in peripheral nerve extracts.

The effect of DNTF could not be blocked with antiserum to NGF or laminin and was partially induced by exposing the cultured dopamine nerve cells to the first fraction from Example 1 for only 2 days, followed by 5 additional days in serum-free medium only. Thus, constant presence of DNTF may not be

necessary to provide an effective level of neuron survival and function.

#### Example 4 - DNTF Activity In Vivo

5           The first fraction from Example 1 has been tested using three different conditions to determine its effects in vivo. Rat sciatic nerve, including the tibial and peroneal branches were stripped of the surrounding epineurium, cut into 5.0 mm segments, and  
10           washed repeatedly in sterile calcium-magnesium free medium containing 0.1% glucose, 100 ug/ml streptomycin and 2.5 ug/ml fungizone. These nerve segments were then loaded into the lumen of sterilized Amicon XM-50 fibers (1.1 mm ID; cut into 4.5 mm lengths), using  
15           polyethylene (PE 60) tubing attached to a 25 gauge needle and a 1.0 cc syringe. The ends of the Amicon fibers were sealed by pinching with heated forceps, and then placed in RPMI 1640 medium supplemented with 10% fetal calf serum, 5% normal horse serum, 2 mM  
20           L-glutamine, 0.45% glucose, 1 mM sodium pyruvate, 50 units per ml penicillin, and 50 ug/ml streptomycin. The nerve-containing tubes were incubated in this solution for 1-2 days in a humid chamber of 95% air, 5% CO<sub>2</sub> at 37°C.

25           The hollow polymer fibers serve as carriers for subsequent transplantation of the nerve segments into the central nervous system. The polymer fibers comprise a semiporous membrane that allows for the exclusion of molecules of specified molecular weights.  
30           The membrane of the polymer fibers used in this experiment allowed the passage of molecules up to 50,000 daltons. The nature of the polymer is such as to inhibit rejection of the transplant by the immune system.

35           The nerve tube implants thus prepared were transplanted into the lateral cerebral ventricular of

young and aging normal rats for 2, 4, 8 or 10 weeks. In addition, the nerve tube implants were transplanted into young adult rats with unilateral lesions of the nigro-striatal dopamine system and co-grafts of fetal dopamine nerve cells (embryonic day 14). Lesions of the nigro-striatal dopamine system result in a well characterized unilateral rotation when the animals are challenged with dopamine agonists such as amphetamine. Transplants of fetal dopamine neurons have been found to reinnervate target areas denervated by the lesions, and to reverse the behavioral rotation.

In brain sections of animals that received only the nerve tube transplants, tyrosine hydroxylase staining was enhanced in the dopamine-containing neurons in the substantia nigra, as well as in nerve fibers located in the target region of these neurons. This effect was observed at 4, 8, and 10 weeks, but not at 2 weeks following transplantation. The presence of enhanced tyrosine hydroxylase staining in neurons distant to the nerve tube implant indicate that DNTF is a soluble factor that is transported specifically in dopamine-containing nerve cells.

Co-grafts of nerve tubes with fetal dopaminergic neurons into animals with unilateral lesions of the nigro-striatal dopamine system resulted in enhanced behavioral recovery as determined using the amphetamine-induced rotation. Immunohistochemical evaluation of the brain revealed enhanced tyrosine hydroxylase staining of grafted neuron cell bodies and axons. Invariably, fibers from the grafted neurons grew in the direction of the nerve tube implant, suggesting some "chemotactic" property. This phenomena is not unexpected, for when a soluble source of a trophic factor is released from the nerve tube, the concentration of the trophic factor will be highest in proximity to the nerve tube, with

concentration decreasing as the distance from the nerve tube increases. In this case, the axons of the grafted nerve cells were attracted toward the gradient containing the highest concentration of trophic support.

The in vivo test data indicate that DNTF treatment in patients having Parkinson's Disease would provide a valuable alternative to present therapy, by facilitating dopamine neuron survival in the substantia nigra, which present therapy is unable to achieve.

While the various aspects of the present invention have been described and exemplified above in terms of certain preferred embodiments, various other embodiments may be apparent to those skilled in the art. For example, while the DNTF of the invention is derivable from cells of the peripheral nervous system according to the isolation and purification procedures described above, it may also be derivable from such cells using recombinant DNA techniques. Thus, it is quite possible that the gene responsible for DNTF expression could be isolated, cloned, and expressed in a suitable host cell to enable large scale production of biologically active DNTF. Since the same gene would be responsible for production of both the "natural" and "recombinant" proteins, their pharmacological effect would be expected to be substantially equivalent. This invention is, therefore, not limited to the embodiments specifically described and exemplified, but is capable of variation and modification, without departing from the scope and spirit of the present invention, as set forth in the following claims.

## WHAT IS CLAIMED IS:

1. A composition of matter for treatment of Parkinson's Disease which comprises a purified and concentrated form of soluble dopaminergic neurotrophic factor derivable from cultured cells of the mammalian peripheral nervous system, said factor having a molecular weight of less than 10,000 daltons, and being capable of increasing the survival time of fetal, non-mitotic dopamine nerve cells in culture, and of increasing in vivo expression of tyrosine hydroxylase in substantia nigra dopamine nerve cells exposed to said factor, but having no appreciable effect on neurite outgrowth, said factor having a neurotrophic effect on substantia nigra dopamine nerve cells.

2. A pharmaceutical preparation for the treatment of Parkinson's Disease which comprises, as an active ingredient, a purified and concentrated form of soluble dopaminergic neurotrophic factor derivable from cultured cells of the mammalian peripheral nervous system, said factor having a molecular weight of less than 10,000 daltons, and being capable of increasing the survival time of fetal, non-mitotic dopamine nerve cells in culture, and of increasing in vivo expression of tyrosine hydroxylase in substantia nigra dopamine nerve cells exposed to said factor, but having no appreciable effect on neurite outgrowth, said factor having a neurotrophic effect on substantia nigra dopamine nerve cells, in an amount effective to facilitate survival of substantia nigra dopamine nerve cells.

3. A pharmaceutical preparation as claimed in claim 2 which includes a biologically acceptable medium.

4. A pharmaceutical preparation as claimed in claim 3 wherein said biologically acceptable medium is a liquid in which said active ingredient is soluble.

5 5. A method for treating patients having Parkinson's Disease, which comprises administering to said patients the pharmaceutical preparation of claim 3.

10 6. A method as claimed in claim 5, wherein said pharmaceutical preparation is administered parenterally.

15 7. A method as claimed in claim 5, wherein said pharmaceutical preparation is administered by introduction into the central nervous system of said patient.

8. A method as claimed in claim 7, wherein said pharmaceutical preparation is administered by intracerebroventricular infusion.

20 9. A method as claimed in claim 5, wherein said pharmaceutical preparation is administered non-parenterally.

10. A method as claimed in claim 9, wherein said pharmaceutical preparation is administered orally.

25 11. A method as claimed in claim 9, wherein said pharmaceutical preparation is administered intra-nasally.

30 12. A method for treating patients having Parkinson's Disease, which comprises transplanting into the striatum of said patients cells of the peripheral nervous system, said cells being capable of releasing soluble dopaminergic neurotrophic factor, said factor having a molecular weight of less than 10,000 daltons and being capable of increasing the survival time of fetal non-mitotic dopamine cells in culture and of increasing in vivo expression of

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tyrosine hydroxylase in substantia nigra dopamine  
nerve cells exposed to said factor, but having no  
appreciable effect on neurite outgrowth, said factor  
having a neurotrophic effect on substantia nigra  
5 dopamine nerve cells.

13. A method as claimed in claim 12, which  
includes cotransplanting dopamine-synthesizing cells  
of the central nervous system with said dopaminergic  
neurotrophic factor-releasing nerve cells.

10 14. A method as claimed in claim 13, wherein  
mesencephalic dopamine synthesizing cells are  
cotransplanted with said dopaminergic neurotrophic  
factor-releasing nerve cells.

15 15. A method for treating patients having  
Parkinson's disease, which comprises transplanting  
into the striatum of said patients dopamine-  
synthesizing cells of the central nervous system and  
administering to said patients the pharmaceutical  
preparation of claim 2.

20 16. A method as claimed in claim 15, wherein  
said pharmaceutical preparation is administered by  
introduction into the central nervous system of said  
patient.

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# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/04470

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 35/30

USCL: 424/570; 530/839, 300; 514/2, 21

## II. FIELDS SEARCHED

Minimum Documentation Searched \*

Classification System

Classification Symbols

U.S. 530/300, 839; 424/570; 514/2, 21

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched \*

AUTOMATED PATENT SYSTEM; DIALOG DATABASE--BIOTECH ABS: CAS: CURRENT  
BIOTECH; MEDLINE; CLINICAL ABS.

## III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category \* Citation of Document, with indication, where appropriate, of the relevant passages Relevant to Claim No. 1 \*

|        |   |               |
|--------|---|---------------|
| X<br>Y | US, A, 4,701,407, (Appel)<br>20 OCTOBER 1987, see entire document.  | 1-11<br>12-16 |
| Y      | Brain Research, Volume 399, Issued 1986, Tomozawa et al<br>"Soluble Striatal Extracts enhance Development of<br>mesencephalic Dopaminergic neurons in vitro," pages<br>111-124, see page 121. | 1-16          |
| Y      | J. Neurosurg., volume 68, Issued 1988, Sladek et al<br>"Nerve-cell grafting in Parkinson's Disease", pages<br>337-351, see pages 346-349.   | 1-16          |

### \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
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- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search \*

06 November 1990

International Searching Authority \*

ISA/US

Date of Mailing of this International Search Report \*

09 JAN 1991

Signature of Authorized Officer \*

Choon P. Koh  
Choon P. Koh

| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) |   |                                    |
|--|---|------------------------------------|
| Category *   | Citation of Document, <sup>1</sup> with indication, where appropriate, of the relevant passages <sup>2</sup>  | Relevant to Claim No. <sup>3</sup> |
| Y  | Dev. Brain Res., volume 24, Issued 1986, Brundin et al 1-16<br>"Intrastriatal grafting of Dopamine-containing<br>Neuronal cell suspensions: Effects of mixing with<br>Target on Non-Target cells", pages 77-84,<br>see page 77. |                                    |

